Adaptive Ecology of *Lotus corniculatus* L. Genotypes: II. Crossing Ability

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ABSTRACT

Birdsfoot trefoil (*Lotus corniculatus* L.) is a widely distributed polymorphic Old-World perennial forage legume found in wild and naturalized populations throughout temperate regions of Europe, Asia Minor, North Africa, and North and South America. Exotic birdsfoot trefoil germplasm has rarely been used for birdsfoot trefoil genetic enhancement, and information about its crossing ability with other exotics and commercial quality germplasm is not available. The objectives of this research were to (i) characterize the crossing ability of 27 exotic birdsfoot trefoil genotypes with two genetically diverse hybridization testers, and (ii) determine if crossing ability among genotypes was related to their genetic background measured by random amplified polymorphic DNA (RAPD) markers and their ecogeographic origins. Crossing ability was determined using reciprocal crosses with one commercial-quality germplasm and one exotic genotype tester. All possible crossing combinations for an eight-genotype subset were also determined. Crossing ability was measured as the percentage of pollinated flowers that set pods, F_1 progeny pollen viability, pod length, and seeds per pod. Self-genotype pod set and pollen viability were not correlated. Intermediate bridge crosses were identified that could potentially overcome specific cross incompatibilities and be used to obtain progeny for any combination of genotypes. Genotype-crossing ability was associated with ecogeographic features of the collecting site, but not with morphologic characteristics. This differs from findings that other genotype morphologic characteristics are associated with ecogeographic origins and genetic similarities based on RAPD markers. Exotic birdsfoot trefoil genotypes can be utilized with commercial-quality germplasm using conventional crossing methods.

Birdsfoot trefoil a perennial, non-bloating, forage legume that has valuable attributes making it a good alternative to other legumes in temperate regions. It is adapted to slightly acidic, droughty, infertile, or wet soils, which do not support other popular forage legumes (Seaney and Henson, 1970; Marten et al., 1987). A detailed review of birdsfoot trefoil phylogeny and its relationship to germplasm utilization has been presented elsewhere (Steiner, 1999).

Wild relatives of domesticated crops can be rich sources of valuable traits. Extensive genetic variation has been reported within birdsfoot trefoil (Chrtková-Zertová, 1973) and may prove to be a valuable source of pest resistance to improve persistence and to reduce seed shattering during seed production (Grant, 1996). The diverse germplasm may be very important for birdsfoot trefoil cultivar development because many of the existing cultivars have been developed from a narrow germplasm base (Steiner and Poklemba, 1994).

Even though plant introductions provide a diverse array of genes, this diversity may not be easily incorporated into commercially adapted materials because of reproductive barriers (Hallauer, 1978). Birdsfoot trefoil generally exhibits poor self-compatibility (Miller, 1969), but autogamous genotypes are available (Steiner, 1993; Steiner and Poklemba, 1994; Steiner and Beuselinck, 2001). Crossing success can vary under greenhouse conditions (McKee, 1949). The inheritance of male sterility in birdsfoot trefoil may involve the cytoplasm and be controlled by several complementary genes (Negri et al., 1989). The percentage of pods set per flower has been used as a measure of crossing ability in birdsfoot trefoil (Miller, 1969).

Knowledge about the crossing ability of birdsfoot trefoil genotypes is scant, and little work has been done to determine its genetic nature through crossing studies (Negri et al., 1989). In addition, it would be valuable to know whether relationships exist among reproductive traits affecting crossing ability in exotic germplasm and its relationship to their ecogeographic origins so that birdsfoot trefoil germplasm collections can be characterized and better utilized. Identifying possible crossing bridges to transfer characters also could facilitate germplasm enhancement where incompatibility among specific genotypes precludes successful hybridization. Also, the genetic relationships among a large number of diverse genotypes and the effects of genotype differences on crossing ability have not been reported.

The objectives of this research were to (i) characterize the crossing ability of 27 exotic birdsfoot trefoil genotypes with one commercial quality genotype and one exotic genotype tester, and (ii) determine whether crossing ability among genotypes was related to their genetic background measured by random amplified polymorphic DNA (RAPD) markers and collecting-site ecogeography.

MATERIALS AND METHODS

Twenty-eight exotic Old-World birdsfoot trefoil accessions and one North American commercial-quality germplasm were selected from the National Plant Germplasm collection (Table 1). The exotic accessions were either wild or landrace cultivars with their inclusion in the study based on collecting-site ecologic diversity (Steiner and Garcia de los Santos, 2001). Original seeds from the collection expedition of most accessions were obtained from the USDA-ARS Plant Introduction Station at Pullman, WA. The seeds were germinated on blue blotter paper in sandwich boxes and transplanted to greenhouse flats after emergence. Fifteen plants of each accession

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Abbreviations: Self pod set, percentage of self-pollinated flowers that set pods; pod set, percentage of pollinated flowers that set pods; RAPD, random amplified polymorphic DNA.

Table 1. Pollen viability and self-compatibility percentages of 29 birdsfoot trefoil genotypes.

Accession number	Genotype identification	Country	Genotype	
			Pollen viability	Self-compatibility
			%	
PI 31276	MOR†	Morocco	60.2	7.4
PI 180171	CZE	Czech Republic	95.1	0.0
PI 227512‡	IRA1	Iran	91.8	0.0
PI 234670	FRA1	France	96.7	40.1
PI 234811	SWI†	Switzerland	68.2	0.0
PI 235525‡	FRA-2†	France	84.9	1.5
PI 251143‡	MAC	Macedonia	97.1	0.0
PI 260268	ETH†	Ethiopia	97.1	94.0
PI 260692‡	ITA-1	Italy	94.6	0.0
PI 267060	POL	Poland	98.6	0.0
PI 290717‡	UK	United Kingdom	96.5	0.0
PI 93-94	GEO-2	Georgia	99.7	13.1
PI 315082‡	KAZ	Kasakstan	97.1	19.3
PI 315454	RUS-1	Russia	94.0	0.0
PI 319021	SPA	Spain	78.7	0.0
PI 319822	NOR-1	Norway	85.1	0.0
PI 319823	NOR-2†	Norway	95.6	10.7
PI 325369	RUS-2†	Russia	95.5	3.6
PI 325379	UKR	Ukraine	94.3	0.0
PI 369278	RUS-3	Russia	74.0	0.0
PI 464682	TUR†	Turkey	93.1	8.0
PI 384882‡	IRA2	Iran	92.4	0.0
PI 419228	GRE-1	Greece	88.4	8.0
PI 419233	GRE-2	Greece	95.1	3.7
PI 430546	RUS-4	Russia	98.4	12.0
PI 93-21	GEO1	Georgia	65.0	0.0
PI 485601	ITA2	Italy	59.7	8.0
PI 494653	ROM	Romania	92.3	10.0
NC-83	USA†	USA	82.2	4.9

† Genotypes used for bi-directional crossing test.
‡ Original collecting site of this accession not available.

were selected at random, inoculated with an appropriate *Rhizobium* strain, and transplanted into individual cells in flats in the greenhouse under 16/8-h (light/dark) conditions at approximately 20°C. Based on visual plant appearance among genotypes within an accession for morphologic uniformity regarding growth habit, leaf shape and color, amount of pubescence, and flowering, a single genotype was chosen and transplanted into 10-cm diam. by 16-cm deep pots. Vegetative cuttings of all 28 genotypes were made to ensure ample numbers of plants to produce flowers for crossing. The plants were fertilized and watered, and pests controlled as needed to maintain active growth. All of the genotypes, except PI 260268 from Ethiopia (ETH) that required vernalization at 2°C for 4 wk, actively flowered at ambient conditions in the greenhouse over a 3-yr period.

Bee sticks (Williams, 1980) were used to collect pollen from inflorescences and a 70% solution of ethanol was used to kill pollen on the bee stick between crosses. The percentage of self-pollinated flowers that set pods (self pod set) for each genotype was determined by using at least 15 florets that were rolled between the fingers with slight pressure (Seaney, 1962). At maturity, which occurred approximately 30 d after pollination, pods were threshed by hand, and the number of fully developed and shriveled seeds was determined. General measures of reproductive success were (i) the percentage of pollinated flowers per umbel that set pods (pod set), (ii) the number of seeds produced per pod set, (iii) pod length, and (iv) the pollen-viability percentage of the resulting F1 progeny from each cross.

Three randomly selected, well-developed florets collected during anthesis from all parental genotypes and from florets of their F1 hybrid progeny were squeezed to exude pollen onto a microscope slide. The pollen was immediately stained with 500 mL of glycerol L^{-1} of water containing acetocarmin (10 g L^{-1}). At least five plants per cross were sampled, and the average pollen viability determined (Beuselinck et al., 1996). The reliability of this technique was verified by comparison with an in vitro pollen-germination method (Kariya, 1989) using a mixture of 20% sucrose, 1% agar, and 20 ppm boric acid. No pollen germination occurred in the absence of boric acid. The pollen-stain and pollen-viability methods were correlated ($r = 0.66$; $P \leq 0.001$).

Crossing ability between 27 exotic genotypes and the NC-83 commercial germplasm (USA) and exotic PI 234811 (SWI) testers was determined using the testers as both pollen and seed parents. The USA genotype had an erect-growth habit and the typical appearance of North American cultivars, whereas SWI had the morphologic appearance of *L. alpinus*. *Lotus alpinus* grows sympatrically with birdsfoot trefoil about 2000 m in the French Alps (Grant, 1999) and easily crosses with birdsfoot trefoil (Somaroo and Grant, 1971). A 15-flower minimum was used for making crosses in both directions using the USA and SWI testers. All crosses, except for ETH as the seed parent, were done without emasculation 1 to 3 d before pollen dehiscence. Flowers from the ETH genotype used as the seed parent were emasculated by removing the 10 stamens and fused keel petals with forceps, leaving intact the standard and two wing petals (Seaney, 1962). Pollen was transferred after 1 d when the emasculated flower was fully expanded.

The USA and SWI testers and 6 others of the 27 exotic genotypes (PI 31276, MOR; PI 235525, FRA-2; PI 260268, ETH; PI 464628, TUR; PI 325369, RUS2; and PI 319823, NOR2) were selected for their genetic and ecologic diversity, and reciprocal crosses were made in all possible combinations to determine their crossing ability. This was done using at least 80 flowers per cross in both directions. All crosses were done as described above for determining the crossing ability with testers.

Analysis of variance was used to determine the effects of genotype, crossing direction, and their interaction on repro-

ductive success. The mean comparisons were done using Fisher's protected least significant difference (LSD) test at $P \leq 0.05$ (Systat 5.2.1 for the Macintosh, Evanston, IL). Notched box plots were used to present summaries of differences between USA and SWI parental testers and crossing direction for the tester pod-set percentage, pod length, seeds per pod, and F1 pollen viability (Statview 512+, Brain Power, Calabasas, CA). The notches represented the 95% confidence bands about the median. Notched box plots were also used to display the results from all possible crosses among the eight genotypes.

Pearson's correlation coefficient (Snedecor and Cochran, 1980) was used to determine associations between parental clone self pod-set and pollen-viability percentages with the ecogeographic variables for the 29 parental clones, and the relationships among the measures of reproductive success with genotype collecting-site ecogeography for the eight clone subset. Only genotypes that set pods were used in the correlation analyses for pod length, seeds per pod, and F1 pollen viability. The ETH genotype was excluded from the correlation analyses of self pod set and pollen viability because it was nearly autogamous.

Four groups of genotypes from a cluster analysis based on random amplified polymorphic DNA (RAPD) bands (Steiner and Garcia de los Santos, 2001) were used as the independent variable in an analysis of variance to determine the effect of genotype classification on pod-set and pollen-viability percentages. Mean separations were based on Fisher's protected LSD test.

Product moment correlations (Mantel, 1967) for the measures of reproductive success with genetic distance among the genotypes based on RAPDs, and aggregate morphologic and ecologic distances were determined using the MXCOMP command of NTSYSpc program, version 2.2 (Rohlf, 1997). Product moment correlations were also determined for the four reproductive-success measures with the 12 monthly averages for low, high, and average temperature, sunshine percentage, snow, and precipitation. Pearson correlations (Snedecor and Cochran, 1980) were used to measure the association of the single measures for collecting-site elevation, latitude, and longitude with each of the four measures of reproductive success.

RESULTS AND DISCUSSION

Characterization of Parental Genotypes

All genotypes had pollen-viability percentages >70% except for MOR, SWI, GEO-1, and ITA-2 (Table 1). The range for the self pod set was from 94% for ETH to 0% for 14 of the 29 genotypes (including the USA tester). The ETH genotype was one of several known wild autogamous accessions of birdsfoot trefoil found in Ethiopia (Steiner and Poklemba, 1994). Those genotypes that were self-compatible had a pod set greater than that reported by MacDonald (1946). Chandler et al. (1986) reported that wild *Helianthus* genotypes with low pollen-viability percentages sometimes indicate that the individuals are actually natural hybrids. Because birdsfoot trefoil comprises many highly variable morphologic forms (Chrtková-Zertová, 1973), it is possible that some of the genotypes used are natural hybrids between different botanical varieties of birdsfoot trefoil. No relationship was found between pod set and pollen viability ($r = 0.20$; $P \leq 0.32$; excluding ETH that was nearly autogamous).

Pod length was positively correlated with the number of seeds per pod, regardless of genotype or crossing direction ($r = 0.77$ and 0.81 for USA seed and pollen parents, respectively, and $r = 0.82$ and 0.81 for SWI seed and pollen parents, respectively; $P \leq 0.001$ for all). The correlations among all other measures of reproductive success were not significant, regardless of genotype or crossing direction.

No relationship was found between pollen viability and pod set of self-pollinated flowers with any of the collecting-site ecologic variables. This finding differs from that for aggregate morphology with aggregate collecting-site ecology, for aggregate morphology with specific ecogeographic features of the collecting sites, and for specific quantitative morphologic traits with collect-

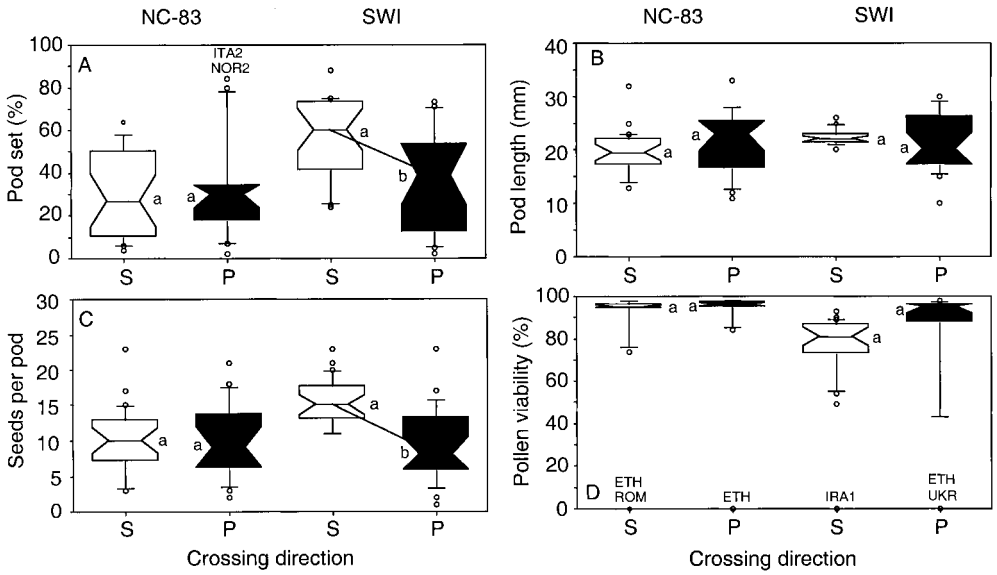

Fig. 1. Seed (S) and pollen (P) parent cross-compatibility of 'NC-83' (USA) and PI 234811 (SWI) with 27 exotic birdsfoot trefoil genotypes measured as A, pod-set percentage; B, pod length; C, seeds per pod; and D, pollen-viability percentage. Notched-box plots labeled with different letters indicate comparisons between male and female parents are different at $P \leq 0.05$ according to Student's t test. The notches represent the 95% confidence bands.

ing-site ecogeographic features where there were significant relationships (Steiner and Garcia de los Santos, 2001). It thus appeared, based on the relatively large number and diverse genetic and ecogeographic range of genotypes used in this experiment, that pollen-viability and self-compatibility percentages in these genotypes were environmentally neutral and distributed randomly.

Genotype Crossing Success with USA and SWI Testers

The average response to crossing direction (as seed parent or pollen parent) of the USA tester with the 28 other genotypes was equal for the four measures of reproductive success (Fig. 1). The SWI pod set and number of seeds per pod were greater when the tester was used as the seed parent than pollen parent ($P \leq 0.0001$). However, F_1 pollen viability was less when SWI was used as the seed parent ($P \leq 0.05$).

Incompatibility was expressed in two ways. For some crosses, incompatibility between genotypes resulted in no pods setting (Table 2). Using USA as the seed parent, pollen from all genotypes except GEO-1 resulted in set pods. All genotypes were compatible with SWI as the seed parent. Ten of the genotypes were not compatible with USA pollen (CZE, FRA-2, GRE-2, IRA-2, KAZ, POL, RUS-1, RUS-3, SPA, and UK) and six were not compatible with SWI pollen (CZE, KAZ, ROM, RUS-4, SPA, and UK). The CZE, KAZ, SPA, and UK genotypes were not compatible with either pollen source. Conversely, some combinations showed good crossing success relative to the rest of the crossing combinations (e.g., NOR-2 and ITA-2 seed parents were very compatible with USA pollen; Fig. 1).

A second kind of incompatibility resulted in crossing combinations that successfully produced pods, but the resulting F_1 progeny did not produce viable pollen. These included the USA tester as the seed parent with ETH and ROM pollen, ETH as the seed parent with USA pollen, SWI as the seed parent with IRA-1 pollen, and ETH and UKR as the seed parents with SWI pollen (Fig. 1). The fact that the exotic genotypes did not show crossing direction differences for most of the measures of reproductive success when crossed with USA indicated that genotype NC-83 was suitable both as a seed or pollen parent. A high degree of reproductive success was obtained (pods set) using both USA and SWI as seed parents (96 and 100%, respectively) (Table 2). However, when using these two testers as pollen parents, fewer successful crosses resulted (63 and 78% for USA and SWI, respectively). This indicated that crossing barriers between the exotic genotypes and the two testers could possibly be overcome, but the optimal crossing direction must be determined to achieve successful hybrids. It can be assumed that similar responses would be observed using different testers.

The characterization of crossing success and the identification of complexes of compatible genotypes presents the possibility of using specific accessions as bridges in a birdsfoot trefoil breeding program (O'Donoughue and Grant, 1988). For example, if traits in the ETH genotype were desired (e.g., autogamy), USA would not be a satisfactory crossing parent because the resulting progeny would be sterile (0% F_1 pollen viability for crosses in

Table 2. Seed (S) and pollen (P) parent cross-compatibility of 'NC-83' (USA) and PI 234811 (SWI) testers crossed with 27 exotic birdsfoot trefoil genotypes measured as pod set and F_1 progeny pollen-viability percentages.

		USA				SWI			
		Pod set		Pollen viability		Pod set		Pollen viability	
Accession number	Identification	S	P	S	P	S	P	S	P
		%							
PI 31276	MOR	17.9	23.1	92.7	94.7	53.8	23.2	49.2	94.8
PI 180171	CZE	45.4	–†	92.9	–	43.7	–	86.8	–
PI 227512	IRA-1	63.6	30.0	95.2	95.7	50.0	42.1	0.0	89.9
PI 234670	FRA-1	10.0	20.0	95.4	83.5	40.0	50.0	89.2	93.0
PI 235525	FRA-2	33.3	–	73.5	–	59.8	1.9	60.2	96.1
PI 251143	MAC	54.5	10.0	96.1	96.9	62.5	38.9	80.8	96.2
PI 260268	ETH	9.3	2.5	0.0	0.0	75.0	5.0	54.1	0.0
PI 260692	ITA-1	63.6	8.3	95.7	97.8	73.3	11.1	81.4	93.0
PI 267060	POL	20.0	–	95.9	–	73.3	20.0	86.3	96.3
PI 290717	UK	58.3	–	97.6	–	25.0	–	87.7	–
PI 93-94	GEO-2	27.3	30.0	97.8	95.4	44.4	70.6	77.7	97.8
PI 315082	KAZ	11.0	–	94.3	–	73.3	–	89.6	–
PI 315454	RUS-1	23.1	–	96.1	–	73.3	11.1	88.2	90.9
PI 319021	SPA	27.3	–	94.2	–	87.5	–	68.1	–
PI 319822	NOR-1	30.0	30.0	96.1	96.4	73.3	60.0	79.4	96.4
PI 319823	NOR-2	6.2	83.7	98.0	98.1	51.9	69.5	88.4	96.9
PI 325369	RUS-2	3.6	48.1	93.7	97.2	43.1	55.5	93.0	97.0
PI 325379	UKR	7.7	30.0	96.0	96.2	23.5	5.5	78.6	0.0
PI 369278	RUS-3	25.0	–	94.7	–	75.0	50.0	82.2	96.2
PI 464682	TUR	43.6	7.0	94.5	91.3	60.0	27.7	76.8	79.2
PI 384882	IRA-2	58.3	–	92.3	–	60.0	40.0	74.8	94.6
PI 419228	GRE-1	50.0	30.0	95.7	97.5	60.0	11.8	74.9	91.0
PI 419233	GRE-2	50.0	–	97.3	–	27.8	26.7	62.0	96.1
PI 430546	RUS-4	27.3	20.0	97.5	96.3	38.9	–	72.4	–
PI 93-21	GEO-1	–	70.0	–	93.4	25.0	52.9	81.1	72.4
PI 485601	ITA-2	6.2	80.0	94.8	96.3	35.7	73.3	83.3	75.4
PI 494653	ROM	9.1	30.0	0.0	94.8	60.0	–	83.1	–

† Indicates no pod set, therefore, no pollen was produced.

Table 3. Means for four measures of reproductive success from eight birdsfoot trefoil seed parents when crossed with the seven other genotypes used as pollen parents.

Genotype	Pod set	Pod length	Seeds per pod	F_1 pollen viability
	%	mm	no.	%
NOR-2	54.4a†	26.3a	12.6ab	90.1
SWI	41.7a	22.7ab	15.9a	78.0
RUS-2	40.1ab	22.9ab	9.3bc	91.3
MOR	24.3bc	19.2bc	8.6bcd	85.5
USA	19.4cd	20.3bc	11.3abc	78.0
TUR	16.3cde	15.0bc	7.6cd	84.1
ETH	5.0de	24.1ab	9.9bc	81.0
FRA-2	1.9e	5.9d	4.6d	64.3

† Means within columns followed by the same letter are not significant at $P \leq 0.05$ level based on Fisher's protected least significant difference test.

either direction) and the pod set would be low (<10%), regardless of crossing direction. On the other hand, the SWI genotype used as the female parent was a successful crossing partner with the ETH genotype (75% pod set) and the resulting F_1 progeny were fertile (54%). Since SWI was compatible with USA, SWI × ETH hybrids may be a likely bridge for ETH into USA. Further studies are needed to determine the effect of F_1 parentage on reproductive success from bridge crosses. Also, the relationship between karyotype and cross compatibility among these genotypes needs to be determined. Birdsfoot trefoil crosses that demonstrate high levels of compatibility may be due to high degrees of chromosome homology (Grant et al., 1962).

Identification of predictors of reproductive success could facilitate exotic germplasm utilization. Based on a four-group classification using RAPDs (Steiner and Garcia de los Santos, 2001), genotypes from Group-4 (GEO-2, ITA-2, and ROM) had the lowest percentage of pods set (5% compared with 33, 29, and 46% for groups 1, 2, and 3, respectively) when USA was the female parent ($P \leq 0.03$) and the highest percentage of pods set (60% compared with 9, 18, and 11%, respectively) when USA was the pollen source ($P \leq 0.01$). Random amplified polymorphic DNA Group-4 genotypes also had the lowest F_1 pollen viability (72% compared with ≈90% for the other three groups; $P \leq 0.04$). These results suggested the possibility of using molecular markers to identify groups of birdsfoot trefoil genotypes that would be cross compatible with specific germplasm sources. Initial molecular marker screening of newly acquired germplasm could predict crossing success before making time-consuming crosses and conducting evaluations.

Table 4. Product moment and Pearson correlations for associations among plant morphologic, genetic, and ecogeographic characteristics of the collecting sites with the means of four measures of reproductive success from eight birdsfoot trefoil genotypes used as the seed parent when crossed with the other seven genotypes used as pollen parents.

Variable	Pod set	Pod length	Seeds per pod	F_1 pollen viability
	Product moment correlation†			
Morphologic	0.19	0.08	0.06	0.12
Genetic	0.12	0.50*	0.12	0.46*
Ecologic	0.55**	0.12	0.59**	0.12
Snow	−0.18	−0.17	0.58**	−0.07
$Temperature_{Ave}$	0.52**	0.23	0.67**	0.19
$Temperature_{Low}$	0.31	0.11	0.14	0.09
$Temperature_{High}$	0.37*	0.22	0.73**	0.16
Sunshine	0.77***	0.09	0.19	0.07
Precipitation	0.49*	0.17	0.20	0.05
	Pearson correlation†			
Elevation	−0.10	0.35	0.35	−0.14
Latitude	0.69*	0.01	0.28	0.59
Longitude	0.06	0.08	−0.17	−0.22

* indicates r at $P \leq 0.05$.
** indicates r at $P \leq 0.01$.
*** indicates r at $P \leq 0.001$.
† The product moment correlation was used when the descriptive variable was composed of more than one measurement. The Pearson correlation was used when the reproductive measures of eight seed parent genotypes were correlated with a single characteristic of the collecting site.

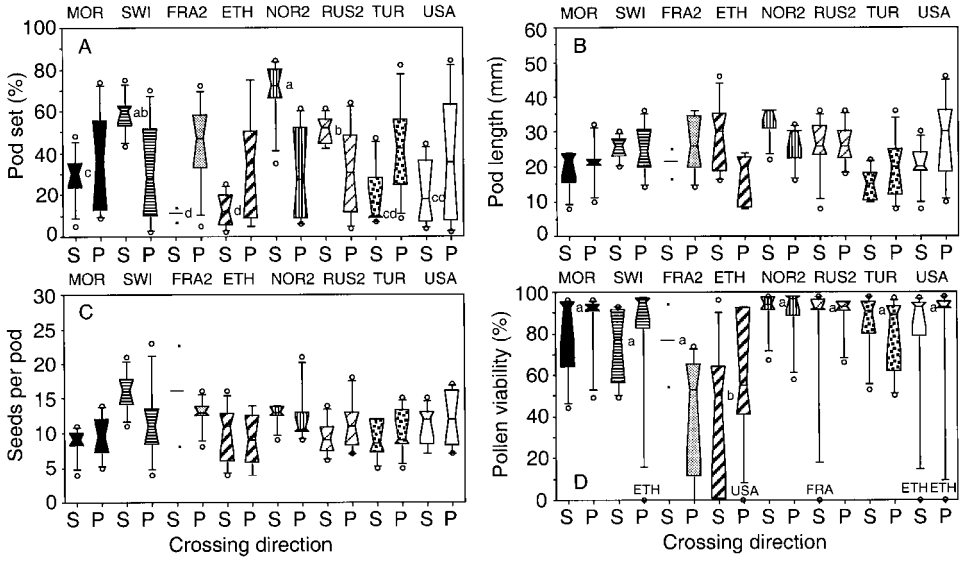

Fig. 2. Seed (S) and pollen (P) parent cross-compatibility among seven exotic and one North American-adapted birdsfoot trefoil genotypes measured as A, pod-set percentage; B, pod length; C, seeds per pod; and D, pollen-viability percentage. Genotypes from accessions used are MOR, PI 31276; SWI, 234811; FRA, PI 235525; ETH, 260268; NOR, PI 319823; RUS, PI 325369; TUR, PI 464682; and USA, 'NC-83'. Notched-box plots labeled with different letters are different at $P \leq 0.05$ according to Student-t test. The notches represent the 95% confidence bands.

Crossing Ability Among Diverse Genotypes

A subset of eight ecologically diverse genotypes, including USA and SWI, were crossed in all possible combinations as both pollen and seed parents (Table 3). Means for pollen parents are not presented because no differences among genotypes were found. There was also no interaction effect between genotype and crossing direction on F_1 progeny pollen viability ($P \leq 0.91$) (Table 3). However, genotype and crossing direction interacted and affected pod-set percentage, pod length, and seeds per pod ($P \leq 0.0001$, 0.0001, and 0.05, respectively; Fig. 2).

The NOR-2 and SWI genotypes were the most cross-compatible genotypes and the FRA-2 the least (Table 3). On the basis of pod set, the NOR, SWI, and RUS genotypes were generally superior as female parents (Fig. 2A). For the TUR and FRA genotypes, the pod set was better when they were used as pollen parents than seed parents. Crossing direction did not affect the pod set of MOR, USA, and ETH. The ETH genotype had the lowest F_1 progeny pollen viability compared with the rest of the genotypes (Fig. 2D). Crossing combinations that resulted in nonviable pollen included SWI as the pollen parent with ETH as the seed parent, ETH as the pollen parent with USA as the seed parent, RUS as the seed parent with FRA as the pollen parent, and USA as both the pollen and seed parent with ETH (Fig. 2D).

Reproductive Success and Genetic/ Ecogeographic Background

No relationship was found among the four measures of reproductive success for the eight genotypes with aggregate morphology (Table 4). However, the pod-set percentage was associated with the aggregate ecology of the collecting sites, average and high temperature, sunshine, precipitation, and latitude. The seeds produced per pod also were strongly associated with specific ecogeographic characteristics of the collecting sites (aggregate ecology, snow, average temperature, and high temperature). Pod length and F_1 pollen viability were associated with the genetic background of the genotypes but not with any ecogeographic characteristics. Unlike pollen viability and self-compatibility of the parental clones, reproductive barriers to cross compatibility in birdsfoot trefoil were related to the ecogeography of the collecting-site habitats and thus were not distributed randomly throughout the species. This finding was similar to the relationship observed with other morphologic characteristics, including both vegetative and reproductive features (Steiner and Garcia de los Santos, 2001).

CONCLUSIONS

The birdsfoot trefoil parental genotypes exhibited a range of pollen-viability and self pod-set percentages; these two responses were not correlated. There was also no relationship between the pollen-viability or self pod-set percentages and any of the ecogeographic variables that described the genotype collecting sites. For crosses between the two hybrid testers and the rest of the genotypes, the only correlated measures of reproductive success were the number of seeds per pod and pod length. Barriers to crossing ability with the testers were expressed either as a failure to set pods or production of F_1 progeny that did not produce viable pollen. However, when poor crossing ability was observed between specific genotypes, intermediate crosses were identified that might bridge any incompatible parent combinations. Random amplified polymorphic DNA markers identified a group of genotypes with the lowest pod-set percentage when USA was the seed parent, the highest pod-set percentage when USA was the pollen source, and the lowest F_1 pollen viability percentage. These results indicated the possibility of using specific markers to identify genotypes that will cross with known germplasm sources. Reproductive barriers to birdsfoot trefoil cross compatibility were related to the ecogeography of the collecting-site habitats and thus were not distributed randomly throughout the species. Exotic birdsfoot trefoil genotypes can be utilized with commercial-quality germplasm using conventional crossing methods.

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Incidence and Diversity of *Neotyphodium* Fungal Endophytes in Tall Fescue from Morocco, Tunisia, and Sardinia

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ABSTRACT

There is a premium on having *Neotyphodium* germplasm available for temperate grass improvement programs because these fungal endophytes present opportunities for developing new grass–endophyte combinations for enhanced tolerance to abiotic and biotic stresses. Unfortunately, surveys have revealed a low incidence of *Neotyphodium* fungi in grass germplasm collections. This research surveyed tall fescue (*Festuca arundinacea* Schreb.) accessions from a 1994 Australian–U.S. plant-collection trip to Morocco, Tunisia, and Italy (Sardinia) for viable *Neotyphodium* fungi and determined whether infected accessions harbor different *Neotyphodium* genotypes. Conidial measurements of isolates cultured on agar and bioassays of the differential survival of bird cherry-oat aphid [*Rhopalosiphum padi* (L.)] on infected accessions were used to characterize *Neotyphodium* diversity. A secondary objective determined the consistency of a polymerase chain reaction (PCR) method to detect *Neotyphodium* fungi in tall fescue. *Neotyphodium* was detected in 336 of 439 plants (76.5%) distributed among 104 accessions, of which 99 were endophyte-infected. Mean conidial lengths of 42 isolates ranged from 3.91 to 9.91 μm. Most of the isolates (71.4%) had conidia with mean lengths smaller than the lower limit (6.5 μm) characteristic of the tall fescue endophyte *N. coenophialum* (Morgan-Jones and Gams) Glenn, Bacon, and Hanlin. In aphid assays, all endophyte-free plants were susceptible to *R. padi* and all but two infected plants were resistant to this aphid. Thus, a Mediterranean plant-collection trip secured diverse *Neotyphodium* endophytes in tall fescue for storage in seed banks, and a PCR assay detected *Neotyphodium* in tall fescue plants of diverse geographical origin.

PLANT EXPLORATION as a primary mechanism for finding and adding new plant germplasm to ex situ repositories is also a means for collecting microbial germplasm in the form of *Neotyphodium* Glenn, Bacon, and Hanlin (formerly *Acremonium*) (Glenn et al., 1996) fungal endophytes of temperate grasses for storage in repositories (Clement et al., 1994). For example, plant exploration missions to North Africa and Sardinia during the early 1990s targeted forage grasses and their associated *Neotyphodium* endophytes for collection and preservation in seed banks (West et al., 1992; Chakroun et al., 1995; Cunningham et al., 1997). These collecting missions aimed to broaden the genetic diversity of forage grasses and *Neotyphodium* endophytes in germplasm collections.

There is widespread interest in *Neotyphodium* endophytes because their presence in temperate grasses is linked to enhanced plant fitness, such as greater drought tolerance and resistance to insect and mammalian herbivores (Clement et al., 1994; West, 1994; Bacon et al., 1997; Latch, 1997). Toxicity to insects and mammals is the result of specific metabolites (alkaloids) produced by the endophytes in association with their hosts (Porter, 1994). A way to overcome the detrimental characteristic of mammalian toxicoses associated with endophyte infection is to produce new grass–endophyte associations with naturally occurring strains of *Neotyphodium* that do not produce mammalian toxins, but do produce the necessary metabolites for insect resistance and other ecological benefits (Rowan and Latch, 1994; Latch, 1997). For such purposes, having a large diversity of *Neotyphodium* endophytes available is as important as having a diversity of forage grass germplasm on hand for plant breeding.

Although conserved seed might be expected to supply the necessary pool of *Neotyphodium* and alkaloid diversity for forage grass and turfgrass improvement programs, surveys of seed banks in Europe and the USA revealed a relatively low incidence of endophyte infection among accessions of *Festuca* (Latch et al., 1987; Springer and Kindler, 1990; Holder et al., 1994; Siegel et al., 1995;

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Abbreviations: PCR, polymerase chain reaction; PDA, potato dextrose agar; SEM, scanning electron microscope; WRPIS, Western Regional Plant Introduction Station.